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Invention: Reaction chambers coated with nucleicacids, methods for the production and use thereof

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INS D1

Reaction chambers coated with nucleic acids, methods for the production and use thereof

INS D2

Description

The invention relates to reaction chambers coated with nucleic acids, methods for the production thereof by coating of the reaction chambers with standard nucleic acids, to a test kit containing a standard strip produced by means of the inventive method, to a set of at least two oligonucleotides appropriate for that purpose, to a carrier nucleic acid as well as to a plurality of application possibilities for the quantitative detection of selected nucleic acids in biological substances.

INS D3

SUB D4

Many fields in clinical research and diagnostics, pharmacological drug testing as well as food analytics, require a precise cognition of concentrations of determined nucleic acids (deoxyribonucleic acid [DNA] or ribonucleic acid [RNA]) in a sample to be analyzed. For the measurement of extremely small analyte concentrations, enzymatic amplification methods are frequently used. Hereby, the following methods are *inter alia* concerned: polymerase chain reaction (PCR, US Patents 4,683,195 and 4,683,202, EP 0 201 184; Hoffmann-La Roche), ligase chain reactions (LCR, Abbott Diagnostics, North Chicago, IL, USA), strand displacement amplification (SDA, Walker et al. [1993], PCR Methods Appl.. 3: 1-6 Becton-Dickinson Research Center) and transcription-mediated amplification (TMA, Gen-Probe Inc., San Diego, CA), by means of which analyte nucleic acid concentrations can be measured with an extremely high sensitivity. A prerequisite for the quantitative use of all of the mentioned technologies is the availability of suitable synthetic or native nucleic acid standards of a precisely defined concentration, which either are used as external, i.e. amplified in parallel assays, or as internal standards (i.e. so-called competitors amplified simultaneously in the same assay). Whereas the preparation of suitable standards is known to the person skilled in the art (Zimmermann and Mannhalter 1996, Biotechniques 21: 268-279, Koehler et al. 1995, Quantitation of

mRNA by polymerase chain reaction - nonradioactive PCR methods, Heidelberg, Springer-Verlag), hitherto unsolved problems of process technology exist with the transfer of these standards into a stable form which is ready for application, said fact being a basic prerequisite for the reproducible measurement of unknown nucleic acid concentrations. Problems exist in particular with the best possible storage of highly diluted nucleic acids. These essentially reside in that in practice, extremely low-concentration standard dilution series are worked with in most cases (approx. 1 - 100000 molecules per reaction assay), which despite of a storage at temperatures between -20° C and -80° C, are often unstable (Koehler et al. 1997, Biotechniques 23: 722-726). This problem is, *inter alia,* so countered that low-concentration nucleic acid dilutions are stabilized in the form, that a defined quantity of a specific carrier nucleic acid is added to the dilution, said carrier nucleic acid featuring a sequence homology to the nucleic acid sequence to be detected which is as small as possible (DE Kant et. al. 1994, Biotechniques 17: 934-942, Koehler et al. 1997, Biotechniques 23: 722-726). However, this does not always lead to success (cf. Figure 1), so that it is generally recommended to carry out all the required dilution steps starting every day anew from a stock solution with a defined concentration. This, however, is connected with the disadvantage that the standards used must be prepared in a labor-intensive manner, that they are subjected to a varying pipetting accuracy, and valuable diluted batches can only be used in part. Thus, costs and time expenditure automatically increase at a simultaneously reduced reproducibility and reliability of the methodology. From the application-technical point of view, the depicted procedure is therewith uneconomical, succumbed to several disturbance factors, and hence inappropriate for diagnostic routine laboratories.

Sub D4 Cont.

D5

It is the object of the present invention to develop vessels and methods by means of which the disadvantages of the methods according to the state of the art can be avoided and which are of simple application and storable for a longer period of time with unchanged quality, and which may be components of a test kit.

This task was solved by coating reaction chambers with defined standard nucleic acid concentrations, comprising the following substeps:

- preparation and purification of suitable adsorbable nucleic acid standards
- determination of the precise concentration of the product by means of high performance liquid chromatography (HPLC), called calibration in the following
- preparation of a dilution series from the calibrated standard with addition of defined quantities of a carrier nucleic acid
- inventive adsorption of the standard / carrier nucleic acid compounds on reaction chambers appropriate for amplification reactions, so that these defined coated chambers usable as standards could be transformed into a form imperishable for prolonged periods of time without impairments of quality.

Another subject matter of the present invention is a set of at least two oligonucleotides, a test kit corresponding to the requirements of a routine laboratory, and a plurality of use possibilities of said method.

The subsequently described invention represents a molecular biological method, being a foundation for a preferably automated quantitative measurement of smallest amounts of analyte nucleic acids in diverse biological materials in conjunction with a previous enzymatic amplification. The inventive method consists in transforming nucleic acids into a form appropriate for the standardization of quantitative enzymatic amplification reactions. Surprisingly, it was found that the inventive method allows for the production of nucleic acid-coated, so-called "ready-to-use" standard reaction chambers, which turned out to be of simple use, which are storable without problems for a prolonged period of time with unchanged quality, and which may be used as components for test kits, and hence better comply with the requirements of routine diagnostic laboratories, in particular in view of automated analyses.

ins. D6

Nucleic acids in the sense of the present invention are single-stranded or double-stranded DNA or RNA or synthetic equivalents of DNA and RNA, as well as DNA, the native deoxythymidine (dT) bases thereof being completely or partially substituted by deoxyuracil (dU). The preparation of suitable nucleic acid standards ensues in a form known to the person skilled in the art, preferably by means of PCR with the use of

specific primer oligonucleotides (Example 1, item 1A-B). As nucleic acid standards, native amplification products prepared enzymically, or synthetic nucleic acids are used, the nucleotide sequence of which is homologous to a sequence to be determined, or is preferably identical or characterized by one or more point mutations, deletions or insertions, which preferably lie outside of the primer or probe bonding points. DNA standards are prepared by means of enzymatic amplification of target sequences, preferably by PCR, whereas RNA fragments may be obtained in a known manner by means of *in vitro* RNA synthesis with the use of RNA polymerases (cf. Example 2, item 2.1., C). The prepared nucleic acid fragments are subsequently subjected to a purification procedure, DNA being preferably cleaned by means of agarose gel electrophoresis and subsequent extraction of the standard nucleic acid from the separating gel (Example 1, item 1B), whereas *in vitro*-prepared RNA is extracted from the *in vitro* synthesis assay in a manner known to the person skilled in the art (cf. Example 2, item 2.1., C). The precise measurement of the concentration of the purified nucleic acid product preferably ensues by means of HPLC (Koehler et al. 1997, Biotechniques 23: 722-726, Example 1, item 2). Subsequently, a dilution series is prepared of the calibrated standard nucleic acid. For diluting DNA standards, a DNA solution is used, which is prepared by preferably transforming the DNA of the lambda phage (e.g. strain: lambda cl 857 Sam 7, 48502 bp, lambda DNA) by means of a 5 x 1-minute lasting ultrasonic treatment into fragments of about 1 - 2 kilobases (kb) (Example 1, item 3; the average fragment length was determined by means of agarose gel electrophoresis). This step is supposed to entail an improved adsorption during the lyophilization process and to contribute to an increased durability of the standard nucleic acid in the reaction chamber. It is also possible to use the lambda DNA unmodified, or to use E.coli tRNA instead of lamda DNA. For the dilution of RNA standards, a transport RNA (tRNA) solution is preferably used (Koehler et al. 1995, Quantitation of mRNA by polymerase chain reaction - nonradioactive PCR methods. Heidelberg, Springer-Verlag, Example 2, item 2.2.).

For the quantification of a measurement parameter, various standard dilutions are prepared (Example 1, item 3; Example 2. item 2.2), which preferably allow for the measurement of the entire physiological or technological concentration range of the

analyte to be measured. Aliquots thereof are used for coating those reaction chambers, wherein the enzymatic nucleic acid amplifications necessary for the establishment for example of an calibration graph, are to take place. Preferably, eight separate reaction chambers are coated with eight different standard dilutions (so-called octet strip). According to the present invention, the coating is so carried out that aliquots of the respective standard nucleic acid dilution supplemented by the carrier nucleic acid are mildly dried directly in the reaction chambers used (Example 1, item 4; Example 2, item 2.3.). In a particularly preferred manner, said lyophilization ensues by means of a vacuum centrifuge or a freeze-drier. In a further embodiment, the drying ensues by means of an equivalent drying method, for example, a method for the superheating-free product drying with the use of microwaves (e.g. distributed via GWE mbH, Leuna). The coated reaction chambers produced - such as described - are characterized in that the adsorbed nucleic acid standards adhere so fixedly on the inner surface of the reaction chamber used for coating, that problem-free shipment via mail may, for example, be guaranteed. In the Figures 1 through 4, it is shown in an exemplary manner, which quality requirements are fulfilled by the nucleic acid standards prepared according to the inventive method. For the practice-relevant test of the products on the basis of the described method, the ABI PRISM™ 7700 Sequence Detection System (PE Applied Biosystems) has been used, which presently is the best, yielding extremely reproducible results. With this detection automate, and using the inventive method, as a principle, highly reproducible calibration graphs comprising correlations > -0.99 may be established (Figure 1). The reaction chambers coated with DNA according to the present invention (Example 1), e.g. the so-called "optical" PCR tubes, are superior to the actual state of the art, since they have a distinctly higher stability as compared to the conventionally used PCR standards (Figure 2), and may be stored even at room temperature for a period longer than one year without loss of quality (Figure 3). Reaction chambers coated with *in vitro*-synthesized RNA, are stable for at least six months, at 20° C, as well as at room temperature (Figure 4, Example 2).

Standing upright in a suitable carrier box receiving at least 96 vessels, the coated reaction chambers are closed with a self-adhesive film (e.g. plastic film or aluminum foil,

parafilm), so as to avoid contamination with foreign nucleic acids during storage and transport. In each case one octet strip closed with a film / foil, hence a strip containing 8 different concentrations of the nucleic acids used for coating, is designated as a "ZeptoStrip".

In said reaction chambers, apart from the calibrated nucleic acids, at least two specific marked or unmarked oligonucleotides acting as primers or probes, as well as further reaction components required for the enzymatic amplification, may be contained in a lyophilized form. Alternatively, reaction chambers used may also solely contain in a lyophilized form these specific oligonucleotides acting as primers or probes, the carrier nucleic acid, and further reaction components required for the enzymatic amplification. The inventive test kits consist of at least one "ZeptoStrip", at least two oligonucleotides and one carrier nucleic acid.

The essence of the invention resides in a combination of known elements and novel approaches interacting with one another, and the novel overall effect of which results in an advantage of use and the desired success, which consists in that ready-to-use standard reaction chambers for the quantitative detection of selected nucleic acids in biological substances are now available.

Thus, subsequent to overcoming the above-mentioned disadvantages of prior art, the inventive method features a series of advantages:

1. A manual or automated transfer of diluted acids used for standardization into the reaction compartments used is superfluous, since these are already present in the reaction chamber in a lyophilized form. The user-friendliness is therewith drastically increased, since the ready-to-use standard strips have only to be taken out and to be inserted in a 96-well carrier plate. Upon addition of the preferably premixed reagents for the subsequent amplification reaction, no further manipulations are still necessary. This simplification hence allows a consequent automatization of quantitative enzymatic reactions.

2. Since henceforth, no pipetting of concentrated standards ensues, a potential contamination source is eliminated, and thus the danger of incorrectly positive results is distinctly diminished.

3. By transferring the standard nucleic acids into a non-aqueous medium, a) a possible, undesired microbial decomposition of the nucleic acids used as standards is restricted or prevented, and b) a storability of even extremely low concentrated nucleic acid for prolonged periods of time is reached even at room temperature (Figure 2 - 4). The advantages facilitate quite drastically the shipment as well as the application of the test kits based on this method.

4. The carrier nucleic acids used prevent an unspecific standard adsorption into the one-way materials used for the preparation of the dilution series, and are - detectable upon addition to a PCR assay - at the same time a powerful "enhancer" for the enzymatic amplification.

Figure 1 shows a typical calibration graph obtained by means of a real-time PCR product measurement at the ABI PRISM™ 7700 Sequence Detection System using mdm-2 (murine double minute-2) nucleic acid standards prepared according to the inventive method (use of a standard strip, i.e. eight different lyophilized mdm-2 standard nucleic acid concentrations) (Example 1, item 5). The calculated correlation coefficient is in this case -0.996.

Figure 2 shows in a graphic form the comparison between the inventive coating method and the use of real-time detection alike Figure 1 and the present state of the art. Conventional, i.e. mdm-2 standard DNA fragments of various concentrations (50, 250, 2500, 10000 and 50000 molecules per PCR assay), stored in an aqueous medium and aliquoted the same test day, supplemented with carrier DNA, were compared with lyophilized standards of equal concentration. Whereas conventionally stored standards in particular of a very low concentration (50 or 250 molecules per assay) are completely decomposed as soon as after 14 days of storage and four times freezing / defrosting cycle, (which is reflected in the illustration by reaching the threshold cycle 40 corresponding to

an amplification capacity 0), no loss is detectable with the use of lyophilized standards (ZeptoStrips), even when lowest nucleic acid concentrations are used.

Figure 3 shows PCR results obtained with "mdm-2-DNA" ZeptoStrips, stored alternatively either at -20° C or at room temperature for a period of one year. It can be recognized that identical PCR results, i.e. C_T-values (i.e. parallel curves), were obtained independent of the storage temperature. It may be inferred from these results that the immobilized mdm-2-DNA remains stable over the entire test period independent of the assay concentration and the storage temperature.

Figure 4 shows TaqMan PCR results obtained with "bcl2-cRNA" ZeptoStrips, which had been stored for a period of 6 months alternatively at -20° C or at room temperature. In analogy to the results for mdm-2-DNA, the immobilized bcl-cRNA as well was stable over the entire test period, independent of the assay concentration and the storage temperature.

The inventive use of the reaction chambers coated with nucleic acids takes place in test kits for the detection of selected nucleic acids in biological substances. The test kits are comprised of at least one octet strip closed with film / foil, of at least two oligonucleotides, as well as of one carrier nucleic acid.

The following Examples serve for the explanation of the invention, without being limited thereto.

Exemplary embodiments

Example 1.

Coating of polypropylene reaction chambers ("optical tubes") with defined concentrations of double-stranded mdm-2 standard DNA

1.1 Preparation of a mdm-2-specific standard DNA fragment by means of PCR

A. cDNA preparation from total RNA, isolated from ADR5000 T-lymphoma cell line
(resistance-selected with 5 μg of Adriamycin per ml of culture medium)

- 1 μg of RNA purified by means of RNAzol™ "B" (Tel-Test, Friendswood, TX, USA) in a 20 μl standard reaction assay consisting of AMV reverse transcriptase buffer (250 mmol/l tris/HCl, pH 8.3; 250 mmol/l KCI, 50 mmol/l $MgCl_2$, 50 mmol/l dithiothreitol, 2.5 mmol/l spermidine), 5 U AMV reverse transcriptase, 0.5 mmol/l of each of the dNTPs (Promega, Madison, WI, USA), 10 U recombinant RNase inhibitor (AGS, Heidelberg, FRG), 200 ng Oligo (dT) (Amersham, Pharmacia Biotech, Uppsala, Sweden), to transcribe in cDNA for one hour at 42° C.

B. PCR amplification and purification of the product

➢ *PCR*

- 2 μl each of aliquot of the prepared cDNA are amplified in six identical 50 μl standard PCR assays consisting of 100 ng of each 3' or 5' primer, respectively; 5 μl 10x Taq polymerase buffer (100 mmol/l tris/HCl, pH 8.3; 500 mmol/l $MgCl_2$, 0.01 % [wt/vol] gelatin), 1.5 U AmpliTaq® polymerase (Norwalk, CT, USA, Perkin-Elmer)m and 8 μl of dNTPs (0.2 mmol/l of each nucleotide with use of dUTP instead of dTTP) in GeneAmp®9600 Thermalcycler (Perkin-Elmer).
- Program: 94° C for 30 s. 55° C for 30 s, 72° C for 1 min
  35 cycles
- Sequences of the amplification primers used (GenBank Accession Code for mdm-2: I25341)

  MDM2PR1 (1245-1264) 5`-GCC.AAG.AAG.ATG.TGA.AAG.AG-3`
  MDM2PR2 (1439-1455) 5`-ACT.GGG.CAG.GGC.TTA.TT-3`
- Length of the amplified DNA fragment: 211 bp

- *Purification of the 211 bp fragment by means of agarose gel electrophoresis*

- Preparation of 1.5 % agarose gel (Easy-Cast™ Minigel, AGS, Heidelberg), gel slots with sextuple comb, filling of the chamber with TAE buffer (submarine gel)
- Pooling and quantitative application of the prepared PCR assay
- Carrying out of the electrophoresis at 100V, for 45 min
- Making ethidium bromide-colored bands visible in UV light, cutting them out as precisely and rapidly as possible by means of a scalpel, transferring into 1.5 ml Eppendorf vessels
- Purification of DNA from gel blocks by means of QIAquick Gel Extraction Kit (Qiagen, Hilten) according to instruction (elution with H_2O)

1.2 Calibration of the standard stock solution by means of HPLC

- *HPLC equipment*

  3-Line Degasser DG-980-50, PU-980 Intelligent HPLC Pump, Low Pressure Gradient Former, UV-975 UV/VIS Detector, AS-950 Intelligent Sampler, Column-Thermostat Jetstream 2 (Jasco Labor und Datentechnik GmbH, Gross-Umstadt, FRG).
- *Stationary phase:*

  TSK DEAE-NPR column (4.6 mm ID, length: 35 mm) and DEAE-NPR precolumn (4.6 mm ID, length: 5 mm) (TosoHaas GmbH, Stuttgart, FRG)
- *Mobile phase:*

  Buffer A: 25 mmol/l tris/HCl, 1 mol/l NaCl; pH 9.0

  Buffer B: 25 mmol/l tris/HCl; pH 9.0
- *HPLC run conditions:*

- Pressure: 80 - 120 bar (maximum 200 bar)
- Flow rate: 1 ml/min
- Temperature: 20° C

- UV detection at 260 nm
- Analyte: supplementation of approx. 10 - 200 ng purified PCR fragment with buffer B on 40 µl, injection of 20 µl per run in each case in double detection
- Standard (separate run): 36 µl of buffer B plus 4 µl of Low Mass DNA Ladder™ (Life Technologies, Gaithersburg, MD, USA), consisting of 6 smooth-ended DNA fragments in the range between 100 and 2000 bp (final concentration: 5 to 100 ng DNA per band), injection of 20 µl per run (double detection)
- Carrying out a discontinuous gradient program for 25 min in the manner described below:
  1. Equilibration of the column with 25 % buffer A in buffer B
  2. 25 % A in B: sample application up to 0.5 min
  3. 25 - 43 % A in B: linear gradient of 0.5 - 4.5 min
  4. 43 - 60 % A in B: linear gradient of 4.5 - 20 min
  5. 60 - 100 % A in B: linear gradient of 20 - 22 min
  6. 100 - 25 % A in B: linear gradient of 22 - 24 min
  7. 25% A in B: equilibration of 24 - 25 min

The data acquisition ensues by means of integration of the peaks by Borwin™ Chromatography Software, version 1.20 (IMBS Developpements, France). For a precise measurement of the concentration of the purified DNA standard, the surface below the individual peaks is assessed. The unknown concentration of the standard nucleic acid is calculated with the calibration graph obtained by means of Low Mass DNA Ladder™.

1.3 Preparation of a standard dilution series with carrier DNA

Carrier nucleic acid:

10 OD lambda (dam+) DNA (from bacteriophage lambda cl857 Sam7, AGS GmbH, Heidelberg), dissolved in 0.5 ml 10 mM tris/HCl, pH 8.0; 1 mM EDTA, is transferred into approx. 1 - 2 kb fragments in intervals of 5 x 1 min and intermediate cooling on ice

by means of an ultrasonic bath (Transsonic T570, Ultrasonics) at maximum power; a 10 ng/ml dilution is prepared thereof (1:100), called lambda DNA in the following.

Preparation of a standard dilution series for mdm-2 (20x conc.)

Standard No.	**Dilution SL (preparation)**	**Final concentration [zmol/assay]**	**Molecules per assay**
1	1: 10^4	419.75	252.773
2	1: $10^4 \rightarrow$ 1:5 (20µl[No.1]+80µl λ)	83.95	50.555
3	1: $10^4 \rightarrow$ 1:10 (10µl [No.1]+90µl λ)	41.986	25.277
4	1: $10^4 \rightarrow$ 1:25 (10µl [No.3]+90µl λ)	16.794	10.111
5	1: $10^5 \rightarrow$ (20µl [No.3]+180µl λ)	4.199	2.528
6	1: $10^6 \rightarrow$ 1:4 (25µl [No.5]+75µl λ)	1.050	632
7	1: $10^6 \rightarrow$ 1:10 (10µl [No.5]+90µl λ)	0.420	253
8	1: $10^6 \rightarrow$ 1:50 (2µl [No.5]+98µl λ)	0.084	51

λ = Lambda-DNA, 10 ng/µl

For the preparation of the ready-to-use standard solutions, 5 µl each of the standard dilution series are pipetted into separate multi twist top vials (Sorenson Bio Science, Salt Lake City, UT, USA; distributor: Carl Roth GmbH: Cat.-No.: 8184.1). For the production of multi-functional strips (i.e. strips which are suited for a sequential or simultaneous quantitative measurement of a plurality of different nucleic acid sequences), further 19 standards (5 µl each per standard nucleic acid) are added and - if required - supplemented up to a final volume of 100 µl with lambda DNA.

1.4 Coating of the reaction chambers

- 5 µl each of the ready-to-use standard solution prepared *sub* item 3, are to be pipetted into 8 different "optical tubes" (Perkin-Elmer, Cat.-No.: N8010935) (in decreasing concentration from position A1 - A8), carrying out this

aliquotation preferably with a pipetting robot (e.g. Biomek, company Beckman) for an improvement of quality.

- Insert amplification vessels in black Eppendorf centrifuge adapters (0.2 ml), and lyophilize the samples for 30 min in a vacuum centrifuge (e.g. Univapo 100 H with Unijet Refrigerated Aspirator, company UniEquip) with the rotor counter-heating on until complete dryness is achieved.

1.5 Testing of the produced strips by means of ABI PRISM™ 7700 Sequence Detection System

Optimized mdm-2 TaqMan™ method:

Program:	2-step-PCR	95° C	110:0 min, subsequently
	40 cycles	95° C	00:15 min
		60° C	01:00 min

Reaction conditions:	60 mM $MgCl_2$
	10 pM primer mdm-2Pr11 and mdm-2Pr21
	2 pmol mdm-2Probe
	50 ng lambda DNA (5µl)
	2.5 U AmpliTaqGold™

dNTPs, buffer from TaqMan™ PCR Core Reagents Kit with AmpliTaq™Gold
assay volume: 50 µl

Primer and probe sequences used (GenBank Accession Code für mdm-2: I25341)

mdm2Pr11 (1295-1318)	5`-GAG.AGT.GTG.GAA.TCT.AGT.TTG.CCC-3`
mdm2Pr21 (1352-1373)	5`-TGC.AAC.CAT.TTT.TAG.GTC.GAC.C-3`
mdm2Probe (1320-1350)	5`-FAM-TTA.ATG.CCA.TTG.AAC.CTT.GTG. TGA.TTT.GTC.A-XT-3`-TAMRA

Example 2.

Coating of polypropylene reaction chambers ("optical tubes") with defined concentrations of bcl2 standard copy RNA (cRNA)

2.1 Preparation of bcl2 standard cRNA, which is sequence-homologous to native bcl2 mRNA

(according to a modified method by: Grassi G., Zentilin L., Tafuro S., Diviacco S., Ventura A., Falaschi A., Giacca M., A rapid procedure for the quantitation of low abundance RNAs by competitive reverse transcription-polymerase chain reaction. Nucleic Acids Res 1994; 22:4547-4549).

A. Preparation of bcl2 template DNA with incorporated T7 promoter

➢ Preparation of a bcl2 target fragment with PCR1

- 100 ng of CCRF ADR5000 cDNA (cf. Example 1.1, A) were amplified such as in Example 1.1, B, in a TRIO-Thermoblock™ 48 Thermal Cycler (Biometra, Göttingen).
  Temperature profile PCR1: 94° C for 30 s, 55° C for 30 s, 72° C for 1 min
  40 cycles

- Sequences of the amplification primers used (GenBank Accession Code für bcl2: M14747)
  BCL2PR1 (3498-3516): 5'-CTT.TTG.CTG.TGG.GGT.TTT.G-3'
  BCL2PR2 (3896-3915): 5'-CTT.CTC.CTT.TTG.GGG.CTT.TT-3'

- Theoretical length of the amplified DNA fragment: 418 bp

➢ *Incorporation of the T7 promoter sequence into the synthesized bcl2 target fragment by means of PCR2*

Table 2/1: Pipetting scheme for PCR2 (preparation of 6 identical assays)

Reaction components	**Assay volume µl**
H_2O (HPLC - pure)	21.5
10x PCR-buffer with 1.5 mM $MgCl_2$ (PE Applied Biosystems)	5
DNTPs (1.25 mM each, Promega/Boehringer Mannheim)	8
Primer bcl2-1T7 (50 ng/µl), diluted with H_2O	6
Primer bcl2Pr2 (50 ng/µl), diluted H_2O	2
bcl2 target product (cf. 2.1. A, 1:1000 diluted with H_2O)	5
AmpliTaq Gold (0,5 U/µl, PE Applied Biosystems)	2.5

- The amplification ensued according to the following temperature profile: once through 95° C for 10 min, 40 cycles 95° C for 30 s, 55° C for 30 sec, 72° C for 1 min, one through 72° C, 5 min, 4° C ∞

- Sequences of the amplification primers used:
  BCL2PR2 (3896-3915) : (see above)
  bcl2-1T7: (underlined sequence: T7 promoter)

5`-cgg.gat.ccg.gat.cct.aat.acg.act.cac.tat.agg.gag.aCT.TTT.GCT.GTG.GGG.TTT.TG-3`

- Theoretical length of the amplified DNA fragment: 455 bp

B. Purification and calibration of the bcl2T7-DNA fragment such as described in Example 1.1. and 1.2

C. *In vitro* cRNA synthesis (preparation of 3 identical assays)

Table 2/2: Pipetting scheme for the preparation of bcl2-cRNA synthesis assays

Reaction components	**Assay volume µl**
RNase-free H_2O (treated with DEPC)	1
10x transcription buffer (Boehringer Mannheim)	2
ATP (20 mM, Amersham Pharmacia)	1
UTP (20 mM, Amersham Pharmacia)	1
GTP (20 mM, Amersham Pharmacia)	1
CTP (20 mM, Amersham Pharmacia)	1
Purified bcl2-T7 dsDNA fragment (6 ng/µl)	10
T7-RNA polymerase (Boehringer Mannheim, 20U/µl)	2
RNase inhibitor (Amersham Pharmacia,10 U/µl)	1

- The incubation of the assays was carried out in a thermoshaker for 1 h at 37° C.
- For increasing the cRNA yield, further 20 U T7 polymerase were added per assay after termination of the incubation, thereafter ensued another incubation for 1 h at 37° C (thermoshaker).
- Subsequently, all three assays were mixed and digested with 60 U DNase I (RNase-free, Boehringer Mannheim) for 50 min at 37° C.
- The purification of the cRNA ensued by means of conventional phenol chloroform isoamyl alcohol (25:24:1) extraction such as described in: Koehler T., Lassner D., Rost A.-K., Thamm B., Pustowoit B., Remke H., Eds.: Quantitation of mRNA by polymerase chain reaction - nonradioactive PCR methods, Springer-Verlag Heidelberg, pages 36 - 37.
- The precipitation of the cRNA was carried out over approx. 3 h at -20° C, thereafter, it was washed three times with 300 µl of 75 % ethanol, then centrifuged, and the pellet was stored over night at -20° C under 100µl of 96 % ethanol.

- The next day, drying of the pellet was carried out in a vacuum centrifuge (Univapo 100 H, UniEquip), the dried pellet was solved in 25 µl DEPC H_2O; the concentration detection ensued in 500 µl quartz cells after an UV 260/280 measurement of 2x 2 µl aliquots.
- The total yield of all three assays amounted to 23 µg cRNA at a ratio of 260/280 > 1.8.

D. Characterization of the synthesized cRNA by means of non-denatured agarose gel electrophoresis

(according to a modified method by: Collins M.L., Zayati C., Detmer J.J., Daly B., Kolberg J.A., Cha T.A., Irvine B.D., Tucker J., Urdea M.S., Preparation and characterization of RNA standards for use in quantitative branched DNA hybridization assays. Anal. Biochem. 1995; 226:120-129).

- A minigel chamber (Easy-Cast™, AGS Heidelberg) was freed from RNase over night by means of 2% Absolve NEF-971G cleaning solution (DuPont), and was subsequently rinsed twice with DEPC H_2O.
- Preparation of the gel: Prepare 1% agarose gel (Qualex-Gold-Agarose, AGS) with 1x TAE buffer (from 50x stock solution with DEPC H_2O) (ethidium bromide (1.6 µl 10 % EtBr] poured in), decuple comb; run buffer: 1x TAE (4 µl EtBr/100ml).
- 4 µl of formamide were added to the purified and re-suspended cRNA (approx. 1 µl per 4 µl of volume), as well as 4 µl of a 0.16 - 1.77 Kb RNA ladder (Gibco BRL), and incubated subsequently at 65° C for 5 min.
- Thereupon ensued a rapid cooling down on ice and a subsequent addition of 1 µl of (RNase-free) gelling buffer per tube; from the finished assay, 4 µl aliquots are pipetted into the test gel slots (marker into the outer slots, cRNA sample into the inner slots).
- The electrophoretic separation was carried out for about 2 h at 80 V in the RNase-free minigel chamber cooled in an ice bath. A buffer circulation was achieved by intermittently moving the run buffer.

- The RNase-free documentation of the results ensued by means of a GelPrint Video Documentation Workstation (MWG-Biotech, Ebersberg) with the use of ONE-Dscan™ Software (Scanalytics, Billerica, MA, USA).
- Opposite the theoretical molecular weight of the synthesized bcl2 cRNA of 418 b stood an experimentally determined molecular weight of approx. 400 b.

2.2. Preparation of a bcl2 cRNA dilution series

The dilution of the purified bcl2 cRNA ensued with E. coli tRNA solution (100 ng/µl DEPC H_2O, Boehringer Mannheim).

Table 2/3: Preparation of a bcl2 cRNA dilution series according to the following scheme:

Standard No.	**cRNA dilution factor**	**RNA concentration (indicated in zmol per 5 µl of diluted solution)**
1	$1:10^3$	1610
2	$1:10^4$	161.05
3	$1:10^5$	16.105
4	$1:10^5 \rightarrow 1:5$	3.22
5	$1:10^6$	1.6105
6	$1:10^6 \rightarrow 1:5$	0.322
7	$1:10^7$	0.16105
8	$1:10^8$	0.016105

2.3 Coating of 96 "optical tubes" (1 plate) with bcl2 standard cRNA-defined concentration

- From each of the bcl2 cRNA dilutions (see Table 2/3), 150 µl were prepared and positioned in a prepared coolable rack of a Biomek®2000 pipetting work station (Beckman Instruments Inc. Fullerton, CA, USA).
- A 96-well carrier plate was charged with 96 "optical tubes" and was equally inserted in the therefor intended position in the work station.
- By means of the robot and using suitable plugged one-way pipetting nozzles, 5 µl each of standard "1" was pipetted into the "optical tubes" of position A1 - A12, of standard "2" into the tubes B1 - B12, of standard "3" into the tubes C1 -C 12, etc.
- The chambers containing standard cRNA were all shock-frozen for 30 min at -80° C, and thereupon, avoiding an intermediate defrosting, immediately lyophilized in a pre-cooled Lyovac GT2 (AMSCO Finn-Aqua GmbH, Huerth) for 1 h.
- The tubes were subsequently manually closed with a self-adhesive foil (e.g. Biomek™ Seal & Sample aluminum foils, Beckman Instruments). The "RNA" ZeptoStrips then were so manufactured that the foil adhering to the tubes was vertically cut, so that continuous strips of the positions A1 - H1, A2 - H2, A3 - H3, etc., were formed, in each case containing one concentration of each of the bcl2 cRNA dilutions.
- From the so prepared 12 strips, 6 were in each case stored over the test period either at -20° C or at room temperature.

2.4. Analysis of the prepared bcl2 cRNA strips by means of ABI PRISM™ 7700 Sequence Detection System

A. Materials:

- 5x TaqMan EZ buffer: 250 mM of bicine, 575 mM of K-acetate, 0.05 mM of EDTA, 300 nM of ROX (6-carboxytetramethyl rhodamine), 40 % of (w/v) glycerol; pH 8.2.
- 25 mM $Mn(OAc)_2$
- dNTPs: dATP (10 mM), dCTP (10 mM), dGTP (10 mM) and dUTP (20 mM) mixed in a volume ratio of 1:1:1:1

B. Sequences of the oligonucleotides used

bcl2Pr1 (3498-3516)	see above
bcl2Pr21 (3572-3591)	5`-GCA.AGT.GCA.GCC.ACA.ATA.CT-3`
bcl2Probe (3547-3568)	5`-FAM-CAG.TTC.TGG.GGC.CAA.GAG.GCT.GTXT-3`-TAMRA

(FAM = 6-carboxyfluorescein, TAMRA = 6-carboxytetramethyl rhodamine)

C. Combined reverse transcription and PCR (RT-PCR) with use of the enzyme rTth polymerase (PE Applied Biosystems)

- On the respective test day, into each of the bcl2 cRNA strips stored alternatively at -20° C or at room temperature, the following reaction components were pipetted (Table 2/4, S = standard No., assay volume: 50 µl)

Table 2/4: PCR3

Reaction component	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**
DEPC-H_2O	10.5	10.5	10.5	10.5	10.5	10.5	10.5	10.5
5x EZ buffer with ROX	10	10	10	10	10	10	10	10
DNTPs for TaqMan	6	6	6	6	6	6	6	6
bcl2Pr1 (50 ng/µl)	1	1	1	1	1	1	1	1
bcl2Pr21 (50 ng/µl)	1	1	1	1	1	1	1	1
bcl2 probe (0,79 pmol/µl)	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
$Mn(OAc)_2$, 25 mM	5	5	5	5	5	5	5	5
RTth-polymerase (PE Applied Biosystems, 2.5 U/µl)	2	2	2	2	2	2	2	2

Temperature profile at the ABI PRISM™ 7700 Sequence Detection System:

	59° C, 30 min (reverse transcription)
	95° C, 5 min
40 cycles	95° C, 15 sec
	59° C, 1 min

- By means of PCR3 (Table 2/4), the stability of the inventive bcl2 cRNA strips was analyzed in each case after 7, 14, 28, 90 and 180 days of storage (at -20° C, as well as at room temperature). The results are summarized in Figure 4.

Legend to the figures

Figure 1:

Characteristic calibration graph established by means of a PCR product measurement at the ABI PRISM™ 7700 Sequence Detection System with use of a "ZeptoStrip" coated with mdm-2 DNA according to the inventive method (cf. Example 1, item 5), r = -0.996.

Figure 2:

Advantages of ZeptoStrips (ZSs) as compared to standard nucleic acids, which had been conventionally stored in an aqueous medium according to the present state of the art. Dashed lines: conventional storage; continuous lines: ZeptoStrip. Legend: Initial number of nucleic acid molecules per PCR assay.

Figure 3:

Storability of mdm-2 DNA ZeptoStrips over a test period of 1 year without loss of quality. Legend: Initial number of nucleic acid molecules per PCR assay. Continuous lines: storage at +25° C, dashed lines: storage at -20° C.

Figure 4:

Stability of "bcl2 cRNA" ZeptoStrips, which had been alternatively stored over a period of 6 months either at -20° C or at room temperature. Represented are the regression lines calculated for each standard concentration with consideration of all measurement values obtained in each case at a storage at -20° C and +25° C. Legend: Concentration of the bcl2 cRNA immobilized in the reaction chamber in zeptomol per assay.